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Parenteral iron treatment induces MCP-1 accumulation in plasma, normal kidneys, and in experimental nephropathy

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Introduction. Monocyte chemoattractant protein-1 (MCP-1) promotes renal inflammation, thereby contributing to acute and chronic nephropathies. Its production is stimulated by oxidative stress. Thus, this study tested whether pro-oxidant iron/carbohydrate complexes, used to treat iron deficiency, induce MCP-1 in renal/extrarenal tissues, in plasma, and in the setting of experimental nephropathy.

Methods. CD-1 mice received 2 mg of intravenous iron [complexed with dextran (iron dextran), sucrose (iron sucrose), or gluconate (iron gluconate)]. Renal MCP-1 and/or its mRNA were measured 3 hours to 7 days post-iron injection. Iron effects on liver, lung, spleen, and heart MCP-1 mRNA, and on peritoneal lavage fluid MCP-1 concentrations were assessed. Iron pretreatment effects on MCP-1 levels in unilaterally obstructed kidneys vs. contralateral kidneys were determined. Finally, iron gluconate's influence on proximal tubule [human kidney-2 (HK-2)] cell MCP-1 levels was assessed.

Results. Iron sucrose (the primary test agent) markedly increased plasma and renal MCP-1 levels. It also induced multiorgan MCP-1 mRNA increments (liver > spleen > kidney > lung > heart). Iron gluconate was more potent than iron sucrose; conversely, iron dextran had no discernible effect. The iron dextran and iron sucrose-induced renal MCP-1 mRNA increments (~4×) were persistent, lasting for at least 3 to 7 days. Iron gluconate raised MCP-1 levels in peritoneal lavage fluid. It also doubled MCP-1 in unilaterally obstructed kidneys (ureteral ligation) without altering contralateral (control kidney) MCP-1 content. Iron gluconate raised HK-2 cell MCP-1, implying a direct proximal tubule effect.

Conclusion. Iron sucrose and iron gluconate (but not iron dextran) can induce MCP-1 generation in renal and extrarenal tissues, possibly via transcriptional events. This may dramatically impact renal disease–induced MCP-1 increments. Finally, iron can increase peritoneal lavage fluid MCP-1 levels. Whether the above changes have implications for renal disease progression, and/or for peritoneal inflammation/peritoneal dialysis efficiency, are issues which may need to be addressed.

Key words: iron sucrose, iron gluconate, iron dextran; obstructive nephropathy, oxidant stress.

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Essentially all forms of progressive renal disease have an inflammatory component, induced in part by chemokines which recruit monocytes, macrophages, and T lymphocytes to sites of tissue injury. Monocyte chemoattractant protein-1 (MCP-1) is widely believed to play a dominant role in this process [1–8]. In addition to inflammatory cell recruitment, MCP-1 may also induce direct tissue damage [9]. For example, MCP-1 has been reported to be profibrotic in vitro [10] and in vivo [11–13], to "activate" endothelial and epithelial cells [14, 15], and to exert procoagulant effects [16]. The pathogenic relevance of these findings is underscored by renal MCP-1 accumulation with acute and chronic kidney diseases [17–23], and by observations that blocking MCP-1 activity can ameliorate the severity of experimental nephropathies [24–27]. Increased urinary MCP-1 excretion has been documented during flares of human autoimmune renal disease [e.g., systemic lupus erythematosus (SLE), vasculitis], and in patients with tubulointerstitial nephropathy [28–31]. This strongly suggests that the above-noted experimental observations have clinical relevance.

Oxidative stress is a potent inducer of MCP-1, an effect which is, at least in part, transcriptionally regulated via activation of nuclear factor-kappaB (NF-κB) [32–34]. Iron containing compounds exert marked pro-oxidant effects [35–39]. Given these considerations, a potentially important clinical issue arises: might intravenous iron administration, a mainstay of anemia management in patients with renal disease, induce MCP-1 and potentially alter disease progression? Supporting such a possibility is the fact that all commonly administered intravenous iron formulations (e.g., iron sucrose, iron gluconate, iron dextrans) have well-documented pro-oxidant effects [37–39]. Therefore, the present study sought to gain insights into the following issues: (*1*) Can intravenous iron administration induce renal MCP-1 generation? (*2*) Because intravenous iron injection causes systemic iron exposure, does multiorgan MCP-1 induction result? (*3*) Might this process impact MCP-1 levels in a diseased kidney? (*4*) Given that not all iron formulations have the same toxicologic profiles [36–39], might different agents exert quantitatively different MCP-1 "stimulatory"

effects? Experiments into these issues form the basis of this report.

METHODS

General experimentation considerations

Male CD-1 mice (25 to 35 g) were obtained from Charles River Laboratories (Wilmington, MA, USA) and maintained under routine vivarium conditions. All experiments described below were performed according to Institutional Animal Care and Use Committees protocols approved at the author's institution. The mice were given free food and water access throughout all studies.

Acute effects of intravenous iron sucrose on plasma and renal cortical MCP-1 levels

The following experiments were conducted to assess whether parenteral iron administration evokes an increase in plasma and renal cortical MCP-1 concentrations. Thirty mice were individually placed into cylindric restraining tubes. Shortly thereafter, 15 mice received a tail vein (intravenous) injection of iron sucrose (2 mg of Fe^{3+}, administered as Venofer) (American Regent Laboratories, Shirley, NY, USA). The remaining 15 mice served as controls and received an equal volume of normal saline (0.1 mL) by tail vein injection. Following iron/saline administration, the mice were returned to their cages. Three hours post-injection, each was deeply anesthetized with pentobarbital (~30 to 40 mg/kg). The mice were then subjected to a midline abdominal incision, and ~0.4 mL of heparinized blood was withdrawn from the inferior vena cava (IVC). Seven mice in each group also underwent unilateral renal resection (tissues processed as described below).

Plasma samples were assayed for MCP-1 using a commercially available enzyme-linked immunosorbent assay (ELISA), as per the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The samples were diluted 1:3 to optimize assay performance and run in triplicate. Resected kidneys were immediately iced, and renal cortical samples were isolated using a sterile razor blade. The tissues were homogenized at 4°C in the presence of protease inhibitors [39]. The protein extracts were assayed in triplicate for MCP-1, applying approximately 250 μg of tissue protein extract per ELISA plate well. A full standard curve, with standards supplied by the manufacturer, was run with each assay. Plasma and renal cortical results were expressed as pg/mL and as pg/mg protein, respectively.

Plasma and renal cortical MCP-1 levels 24 hours post-iron sucrose injection

The above experiment was repeated in 14 mice (seven injected with 2 mg elemental iron, as iron sucrose and seven injected with saline). However, plasma sampling was completed 24 hours later. In addition, four mice in each group had renal cortical tissues resected for MCP-1 assay. Analyses were performed as noted above.

Effect of intravenous iron sucrose on multiorgan MCP-1 mRNA

The following experiment had two purposes: first, to determine whether iron-induced increases in plasma and renal cortical MCP-1 (see **Results** section) were associated with increments in renal MCP-1 mRNA expression (suggesting increased transcription); and second, to determine potential extrarenal tissues which might upregulate MCP-1 mRNA in response to intravenous iron sucrose (and hence, potentially contribute to elevated plasma MCP-1 levels). Eight mice were injected with iron sucrose or with an equivalent volume of saline (controls) ($N = 8$), as noted above. Three hours post-injection, they were deeply anesthetized and pieces of the following organs were quickly resected and placed on ice: (*1*) kidney (cortex); (*2*) liver; (*3*) lung; (*4*) spleen; and (*5*) heart [39]. Tissue samples were immediately placed into TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), total RNA was extracted as per the manufacturer's instructions, and the final RNA pellet was brought up in RNase-free water to a concentration of ~1 to 2 μg/mL. RNA integrity was confirmed on an ethidium bromide agarose gel [39]. Reverse transcription-polymerase chain reaction (RT-PCR) were performed using the First-Strand Synthesis Kit for RT-PCR (Ambion Inc., Austin, TX, USA), as previously described [38, 39]. The specific primers for mouse MCP-1 and glyceraldehyde-3-phophate dehydrogenase (GAPDH) (used as the housekeeping gene) were designed with 50% to 60% GC composition (see Table 1). Multiplex PCR was possible because the primers had similar annealing temperatures but dissimilar sizes of the two PCR products. PCR conditions are presented in Table 1 and were optimized for each tissue. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining and quantified by densitometry (Typhoon 8600 scanner) (Amersham Pharmacia BioSciences, Piscataway, NJ, USA). MCP-1 bands were expressed as ratios to simultaneously obtained GAPDH bands.

Comparative trial of different Fe^{3+} formulations: Effects on MCP-1 expression

In previous studies, differential cytotoxicity has been noted with different iron preparations, with iron sucrose being the most toxic [36–39]. The following experiment was undertaken to ascertain whether differential toxicity could also be observed with differing iron preparations using MCP-1 protein/mRNA as the test end points.

Table 1. Primers/conditions used for mouse monocyte chemoattractant protein-1 (MCP-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) analysis by reverse transcription-polymerase chain reaction (RT-PCR)

Genes	Primer sequences	PCR conditions	Product size
Mouse MCP-1	5′-TCA CCT GCT GCT ACT CAT TCA CCA-3′ 5′-AAA GGT GCT GAA GAC CCT AGG GCA-3′	94°C, 45 sec, 59°C, 45 sec 72°C, 45 sec, 23 to 34 cycles	250 bp
Mouse GAPDH	5′-CTG CCA TTT GCA GTG GCA AAG TGG-3′ 5′-TTG TCA TGG ATG ACC TTG GCC AGG-3′	94°C, 45 sec, 59°C, 45 sec 72°C, 45sec, 18 to 26 cycles	437 bp

All renal cortical and hepatic samples were run with 29 cycles for MCP-1 and 18 cycles for GAPDH. To optimize for cardiac evaluation, 34 MCP-1 cycles and 23 GAPDH cycles were employed. Lung and spleen were each evaluated with 34 cycles of MCP-1 and 23 or 26 GAPDH cycles (for lung and spleen, respectively).

Plasma MCP-1 analysis. Mice were placed in cylindric restrainers and injected with either saline ($N = 13$) or with 2 mg of elemental Fe^{3+} in the form of iron sucrose ($N = 13$), iron gluconate ($N = 13$), or iron dextran ($N = 7$). The iron gluconate and iron dextran preparations were obtained from Watson Pharmaceuticals (Morristown, NJ, USA). Three hours later, the mice were anesthetized, plasma was obtained, and then assayed for MCP-1 by ELISA, as above.

Renal cortical MCP-1 MRNA analysis. To further assess possible differential effects of the test iron compounds, renal cortical mRNA levels following their injections were assessed. Mice were injected with either iron sucrose ($N = 7$), iron gluconate ($N = 6$), or iron dextran ($N = 6$). Eight normal mice, injected via the tail vein with 0.1 mL saline, served as controls. Three hours later, the mice were anesthetized, one kidney per mouse was resected, followed by RNA extraction. Renal cortical samples were obtained and analyzed for MCP-1 and GAPDH mRNA, as noted above.

Comparison of hepatic MCP-1 mRNA responses to different iron preparations

Given that the liver is the dominant site for iron carbohydrate complex removal from the circulation [40], the relative toxicities of the different iron preparations within liver was gauged by measuring possible intrahepatic induction of MCP-1 mRNA. Twenty mice were injected with either saline (controls), or with 2 mg of elemental iron in the form of iron sucrose, iron gluconate, or iron dextran ($N = 5$ each). Three hours post-injection, hepatic tissues were removed and assayed for MCP-1 mRNA (as noted above and as detailed in Table 1).

Assessments of renal and hepatic MCP-1 mRNA at 3 days post-iron sucrose injection

Given that each of the above described mRNA analyses utilized a 3-hour post-iron injection end point, the following experiment was undertaken to ascertain whether the 3-hour results simply represented a transient, rather than a sustained, biologic response. Toward this end, mice were injected with saline or iron sucrose ($N = 5$ each). After 72 hours, they were anesthetized, the kidneys and livers were resected and assayed for MCP-1 mRNA.

Assessments of renal and hepatic MCP-1 mRNA at 7 days post-iron gluconate injection

To further explore durability of tissue responsiveness to parenteral iron administration, mice were injected with iron gluconate or intravenous saline ($N = 6$ each). Seven days later, hepatic and renal cortical mRNA values were assessed as above (iron gluconate, rather than iron sucrose, was used to test for durability of response to a second iron compound).

Effect of intravenous iron on peritoneal MCP-1 levels

The following experiment assessed whether intravenous iron therapy might increase peritoneal MCP-1 levels, a result which could theoretically contribute to peritoneal inflammation (and possibly alter the efficiency of peritoneal dialysis). To gain initial insights into this issue, five mice received a tail vein injection of iron gluconate (2 mg Fe^{3+}); five control mice received sham tail vein (saline) injections. Eighteen hours later, the mice were anesthetized and then 1 mL of normal saline was instilled into the peritoneal cavity of each mouse via a transcutaneous injection. The abdominal wall of each mouse was gently massaged to distribute the injected fluid throughout the abdominal cavity. Thirty minutes later, the peritoneal cavity of each mouse was opened through a midline abdominal incision, and a peritoneal fluid sample was drawn into a syringe. The samples from the control and iron gluconate–treated mice were assayed for MCP-1 protein, as noted above.

Iron effects on MCP-1 expression in the setting of acute obstructive nephropathy

The following experiment was undertaken to ascertain whether parenteral iron exposure might impact MCP-1 generation in the setting of a model of progressive nephropathy. To this end, six mice received 2 mg of intravenous iron gluconate and six received a control injection (equal volume of saline). Approximately 18 hours later, each mouse was anesthetized, the abdominal cavity was opened through a midline incision, and the left ureter was ligated at ~0.5 cm below the renal pedicle. The right kidney was left unperturbed. The abdominal incision was then closed with two suture layers (musculature and skin), and then the mice were allowed to

recover from anesthesia. Free food and water access was provided. Approximately 24 hours later (or ~ 42 hours post-iron gluconate or saline injection), the mice were re-anesthetized and both the obstructed and unobstructed kidneys were resected. The cortices were isolated, protein extracts prepared, and these were assayed for MCP-1, as noted above.

Human kidney-2 (HK-2) cell culture experiments

The following experiment was undertaken to ascertain whether proximal tubular cells can directly respond to iron carbohydrate complexes with an increase in MCP-1 generation (if so, this would suggest that the changes observed in renal cortex could have arisen, at least in part, from proximal tubular cell events). To this end, human proximal tubule (HK-2) cells were seeded into eight T75 Costar flasks and maintained in keratinocyte serum-free medium to which was added 2 mmol/L glutamine, 5ng/mL epidermal growth factor (EGF), 40 μg/mL bovine pituitary extract, 25 U/mL penicillin, and 25 μg/mL streptomycin, as previously described [41]. Upon reaching near confluence, iron gluconate (250 μg iron/mL) was added to four of the flasks (an iron dose which does not induce HK-2 cell death) [38, 39]. The remaining four flasks had no iron addition and serving as controls. After an 18-hour incubation, the culture medium was removed, the cells were detached from the flasks with a rubber policeman, and then pelleted by centrifugation. After washing the pellet ×3 with Hank's balanced salt solution (HBSS + Ca^{2+}/Mg^{2+}), they underwent protein extraction in the presence of protease inhibitors [37]. The samples were then assayed for human MCP-1 by ELISA (performed by the Shared Cytokine Resource Laboratory at the authors' institution). The results were expressed as pg/mg cell protein.

Calculations and statistics

All values are presented as means ± 1 SEM. Statistical comparisons were performed by unpaired Student *t* test. If multiple comparisons were made, the Bonferroni correction was applied. Significance was judged by a *P* value of <0.05.

RESULTS

Plasma and renal cortical MCP-1 levels following intravenous iron sucrose injection

Assessments performed at 3 hours post-intravenous iron sucrose injection. By 3 hours post-iron sucrose injection, an approximate threefold increase in plasma MCP-1 levels was observed, compared to saline matched controls (Fig. 1, left). Iron sucrose also induced an approximate 2× increase in renal cortical MCP-1 levels (Fig. 1, right).

Table 2. Monocyte chemoattractant protein-1 (MCP-1) mRNA in mouse organs 3 hours post-intravenous iron sucrose treatment

Organ	Kidney	Liver	Lung	Spleen	Heart
Controls	1.2 ± 0.1	3.1 ± 0.6	1.1 ± 0.6	0.7 ± 0.2	1.4 ± 0.1
Iron sucrose	2.0 ± 0.3	11.3 ± 1.1	1.6 ± 0.2	1.4 ± 0.1	1.7 ± 0.2
P value	<0.025	<0.001	<0.04	<0.001	NS

MCP-1 mRNA values were factored by simultaneously obtained glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values, as assessed by reverse transcription-polymerase chain reaction (RT-PCR). Statistical analyses compare results in each organ. Iron sucrose induced significant increases in all organs, except the heart, with the rank order of increases, as follows: liver > spleen > kidney > lung > heart. Thus, these results indicated that iron sucrose administration induces widespread increases in MCP-1 mRNA, with the degree of increase occurring in an organ-dependent fashion.

Evauation 24 hours post-iron sucrose injection. When evaluations were conducted at 24 hours' post-iron sucrose injections, plasma MCP-1 values were approximately twice as high in iron sucrose–treated mice, compared to their time-matched controls (Fig. 1, left). The corresponding 24-hour renal cortical assessments also demonstrated increased MCP-1 concentrations, compared to time-matched controls (Fig. 1, right) ($P < 0.01$).

Multiorgan MCP-1 mRNA analyses: Three hours post-iron sucrose administration

By 3 hours post-iron sucrose injection, MCP-1 mRNA levels were significantly higher in kidney, liver, lung, and spleen, compared to values in sham-treated controls (values given in Table 2). The cardiac mRNA levels also appeared higher with iron sucrose, but this difference did not achieve statistical significance ($P < 0.08$). When the iron sucrose–induced mRNA data were expressed as% increases over control organ values, the rank order of mRNA increases were as follows: liver (365%) > spleen (100%) > kidney (67%) > lung (45%) > heart (27%).

Comparison of different iron preparations on plasma MCP-1 concentrations

As shown in Figure 2, iron dextran caused no increase in plasma MCP-1 concentrations, compared to simultaneously injected saline controls. Conversely, iron sucrose and iron gluconate approximately doubled and quadrupled plasma MCP-1 levels, respectively (controls, iron sucrose, and iron gluconate all statistically different) (Fig. 2). Thus, clear differences in MCP-1 generation existed with the three test agents.

Comparison of renal MCP-1 mRNA responses to intravenous iron injection

As shown in Figure 3, iron dextran did not increase renal cortical MCP-1 mRNA levels (consistent with the lack of plasma MCP-1 increases with this test agent). In contrast, iron sucrose and iron gluconate each caused statistically significant increases in MCP-1 mRNA, and in a

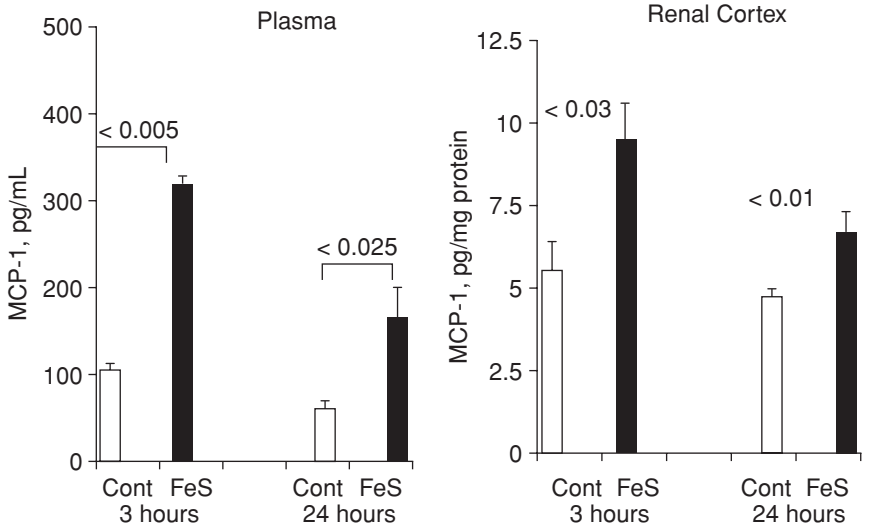

Fig. 1. Plasma and renal cortical monocyte chemoattractant protein-1 (MCP-1) concentrations 3 and 24 hours post-iron sucrose (FeS) injection. (*Left*) Plasma MCP-1 levels were significantly elevated over control (Cont) plasma values at both 3 and 24 hours post iron sucrose injection. (*Right*) Renal cortical MCP-1 levels were also significantly elevated at both time points.

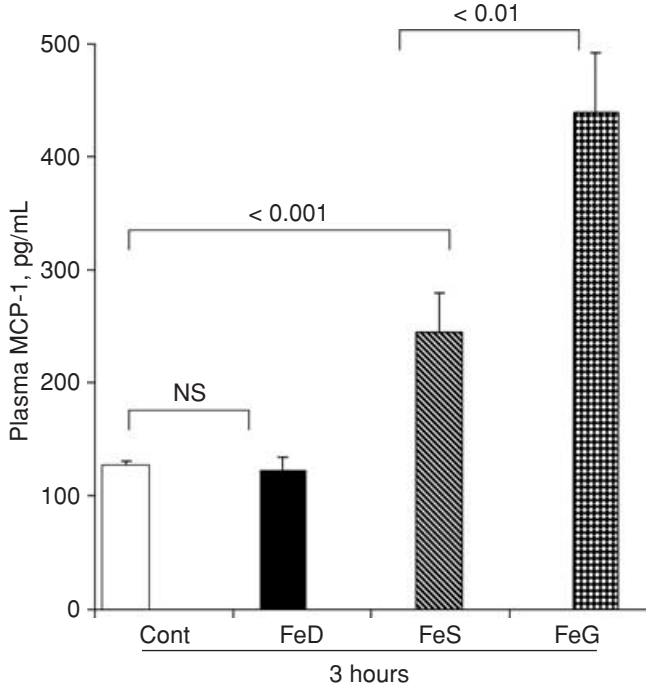

Fig. 2. Comparison of different iron preparations on plasma monocyte chemoattractant protein-1 (MCP-1) levels 3 hours post-injection. Iron dextran (FeD) did not increase plasma MCP-1 levels above control (Cont) values. Conversely, both iron sucrose (FeS) and iron gluconate (FeG) caused an approximate doubling and quadrupling of plasma MCP-1, respectively, with both results being statistically different from the controls and from each other.

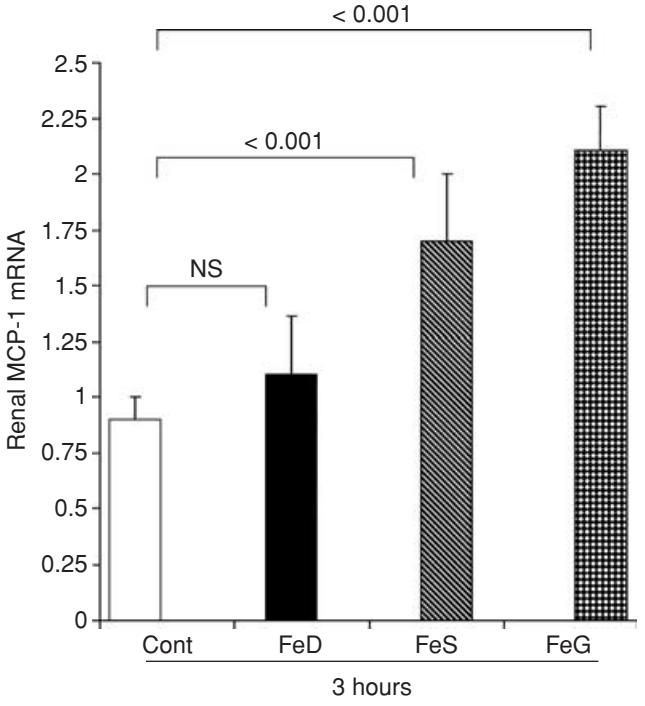

Fig. 3. Renal cortical monocyte chemoattractant protein-1 (MCP-1) mRNA levels at 3 hours post-iron dextran (FeD), iron sucrose (FeS), or iron gluconate (FeG) injection. Iron dextran did not significantly alter MCP-1 mRNA levels vs. control (Cont) values. Conversely, iron sucrose and iron gluconate each raised renal MCP-1 mRNA, with the relative pattern of elevations mimicking the plasma MCP-1 increments, as shown in Figure 2.

relative pattern which was highly similar to the previously noted plasma MCP-1 increases (iron gluconate > iron sucrose > iron dextran = controls; all assessments made at 3 hours post-iron injections) (Fig. 3). Of note, although the increase in MCP-1 mRNA appeared greater with iron gluconate, compared to iron sucrose, this difference did not achieve statistical significance.

Comparison of hepatic MCP-1 mRNA responses to different iron preparations

The 3-hour hepatic MCP-1 mRNA responses to each of the test iron injections are presented in Figure 4. The results were highly congruent with the renal cortical results, described immediately above: (1) iron dextran did

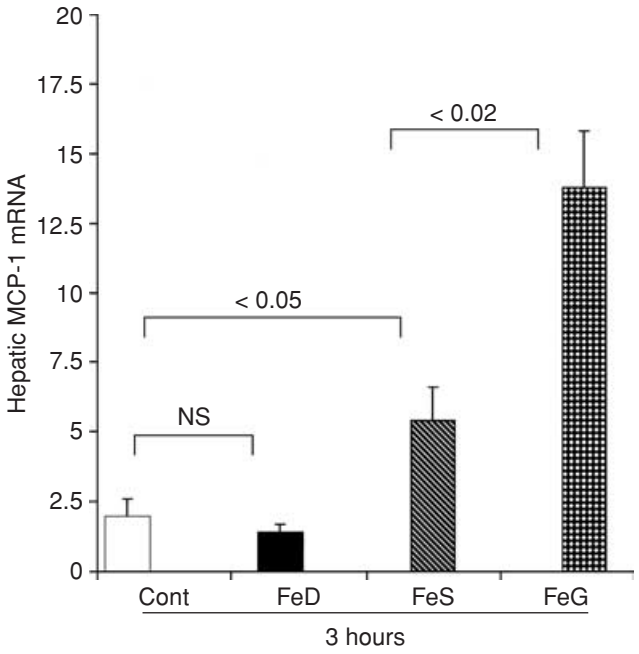

Fig. 4. Hepatic monocyte chemoattractant protein-1 (MCP-1) mRNA levels 3 hours following iron dextran (FeD), iron sucrose (FeS), or iron gluconate (FeG) injections. Iron dextran failed to increase hepatic MCP-1 mRNA levels over control values. Conversely, iron sucrose and iron gluconate each induced marked mRNA increments, both of which significantly differed from control (Cont) values and from each other. Thus, the plasma MCP-1 levels, the renal MCP-1 mRNA levels, and the hepatic mRNA levels each followed the same pattern: iron gluconate > iron sucrose > iron destran = controls.

not raise MCP-1 mRNA, compared to controls; (2) iron sucrose and iron gluconate each caused significant MCP-1 mRNA induction; and (3) the mRNA increases were greater with iron gluconate, compared to iron sucrose ($P < 0.02$). Thus, the plasma MCP-1 levels, renal MCP mRNA levels, and hepatic MCP-1 mRNA levels, each assessed at 3 hours post-iron injection, manifested the same relative toxicity profile: iron gluconate > iron sucrose > iron dextran = control values.

Renal and hepatic MCP-1 mRNA assessments at 3 days post-iron sucrose injection

At 3 days post-iron sucrose injection, an approximate fivefold increase in renal MCP-1 mRNA was observed, compared to values seen in time-matched controls (see Fig. 5, left) [of note, the renal MCP-1 mRNA values were higher at the 3 day (5× increase) vs. the 3-hour post-injection time point (2× increase)]. The liver also manifested a significant increase in MCP-1 message at 3 days' post-iron sucrose injection (3× over baseline) (Fig. 5, left). Thus, these 3-day post-iron sucrose assessments, performed in kidney and liver, indicated that iron sucrose had induced a durable, rather than a rapidly transient, MCP-1 response.

Renal and hepatic MCP-1 mRNA assessments at 7 days post-iron gluconate injection

As shown in Figure 5, right, by 7 days post-iron gluconate injection, an approximate 4× increase in *renal* cortical MCP-1 mRNA values was observed. Thus, no diminution of renal MCP-1 mRNA responses developed between 3 hours and 7 days post-iron gluconate injection (comparing values in Figs. 3 and 5). In contrast to kidney, only a minimal increase in *hepatic* mRNA was observed at the 7-day time point ($P < 0.08$).

Effect of intravenous iron on peritoneal MCP-1 levels

Instillation of saline into the peritoneal cavity allowed for MCP-1 detection in all mice so tested. The values were ~2.5× higher in mice pretreated with iron gluconate 18 hours prior to peritoneal fluid collection (intravenous iron 25 ± 5 pg/mL and controls 10 ± 3 pg/mL) ($P < 0.04$).

Effects of intravenous iron on MCP-1 expression in acute obstructive nephropathy

As shown in Figure 6, iron gluconate pretreatment did not significantly alter MCP-1 levels in nonobstructed right kidneys. Unilateral obstruction, per se, caused an approximate 35% increase in MCP-1 levels (rising from ~8 pg/mg to 11 pg/mg protein, or an increase of 3 pg/mg). Pretreatment with iron gluconate caused the unilaterally obstructed kidney to develop a greatly exaggerated MCP-1 response (rising from 11 to 23 pg/mg protein, or an increase of 12 pg/mg protein). Thus, the obstruction-induced increase in MCP-1 was increased by ~fourfold by iron gluconate pretreatment.

HK-2 cell experiments: Effect of iron gluconate on MCP-1 expression

Iron gluconate addition to HK-2 cells caused an increase in MCP-1 protein levels, compared to coincubated controls (iron addition 52 ± 4 pg/mg protein and controls 38 ± 3 pg/mg protein) ($P < 0.035$). This suggests that renal proximal tubular cells can respond to iron gluconate with an MCP-1 response, potentially contributing to the above-observed increases in renal cortical MCP-1 following intravenous iron injections.

DISCUSSION

The use of parenteral iron, when combined with erythropoietin therapy, is a mainstay of anemia management in patients with end-stage renal disease (ESRD). The resulting improvement of anemia has been widely credited in enhancing patient "well being," and possibly, decreasing morbidity and mortality [42, 43]. Given these benefits, iron therapy is being extended to include patients with chronic nephropathies (i.e., prior to the need

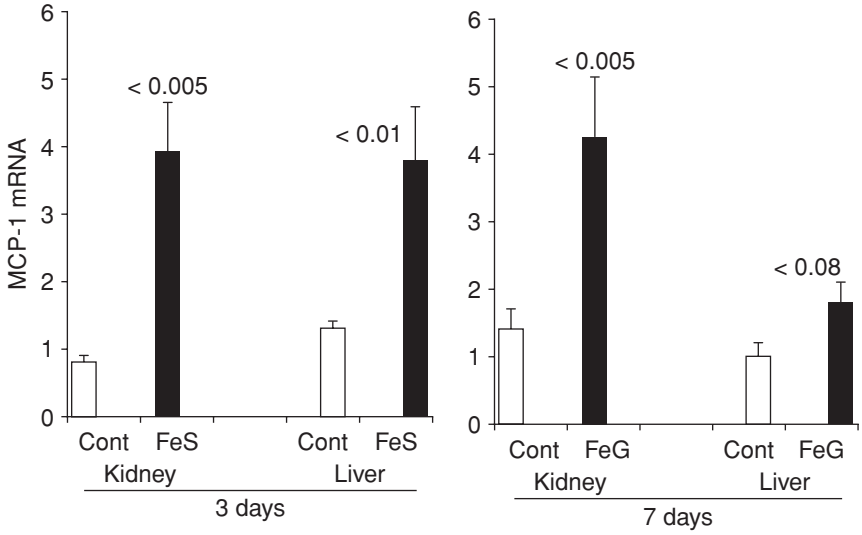

Fig. 5. Monocyte chemoattractant protein-1 (MCP-1) mRNA levels in kidney and liver at 3 days post-iron sucrose (FeS) injection and at 7 days post-iron gluconate (FeG) injection. (*Left*) At 3 days post-iron sucrose injection, dramatic increases in both renal and hepatic MCP-1 mRNA values were observed over control (Cont) values. (*Right*) The ability of iron gluconate to increase renal MCP-1 mRNA at 7 days post-injection indicates the durability of parenteral iron-mediated stimulation of the MCP-1 axis. At 7 days, the renal increase in mRNA was much more striking than was the hepatic mRNA (which failed to achieve statistical significance by two-tailed *t* test) ($P < 0.04$ by one-tailed *t* test).

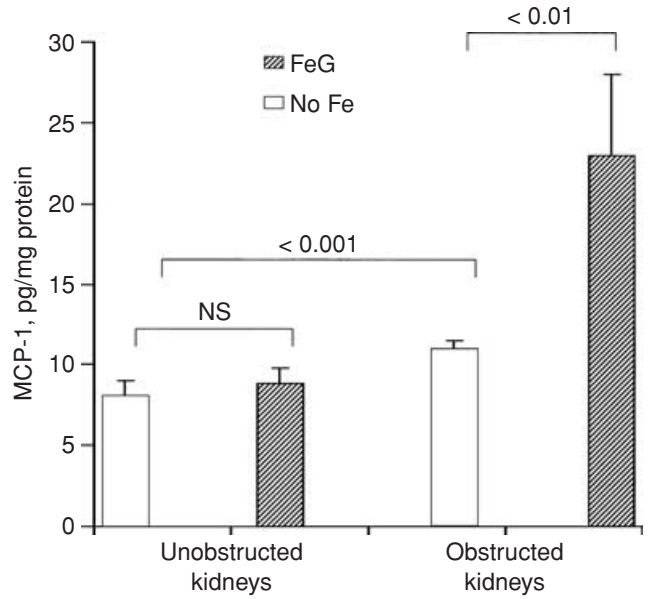

Fig. 6. Monocyte chemoattract protein-1 (MCP-1) protein concentrations in renal cortical tissues in mice which had undergone unilateral ureteral ligation 24 hours earlier. Half of the mice had been pretreated with iron gluconate (FeG) 18 hours prior to the ureteral ligation; the remaining mice had received sham iron (Fe) (saline) injections. The non obstructed kidneys showed no difference in MCP-1 protein levels whether or not they had been pretreated with iron gluconate (~42 hours prior to tissue sampling). Ureteral obstruction caused a mild increase in renal cortical MCP-1 protein in the absence of iron treatment (rising from ~8 to ~11 pg/mg protein) ($P < 0.001$). In contrast, mice pretreated with iron gluconate and then subjected to ureteral obstruction developed dramatic MCP-1 protein increases, more than doubling the values which were observed in obstructed kidneys from noniron gluconate pretreated mice.

for renal replacement therapy). There is an expansive experimental literature which supports a role for intracellular iron (e.g., released from mitochondrial transport proteins and from cytochrome P-450s) as a mediator of acute and chronic renal disease [44–46]. However, whether carbohydrate complexed iron can exert analogous adverse effects remains unknown. Recent experimental data from this laboratory have demonstrated that parenterally administered iron compounds can gain glomerular and intratubular cell access, presumably a prerequisite for inducing cytotoxicity [37–39]. Thus, the potential for protean toxicologic effects would seem to exist.

Given that oxidative stress has been demonstrated to up-regulate MCP-1 expression, and given irrefutable evidence that MCP-1 is an important mediator of diverse forms of renal disease, the overall goal of this project was to ascertain whether parenteral iron administration might impact this redox-sensitive pathway. The results of the above described experiments clearly indicate that the answer is yes. As shown in Figure 1, iron sucrose, perhaps the most widely used iron formulation, increased both *plasma* and *renal cortical* MCP-1 concentrations, assessed at either 3 or 24 hours post-injection. This response was not iron sucrose specific, given that iron gluconate caused even more robust plasma MCP-1 elevations (Fig. 2). Because oxidative stress is known to increase MCP-1 transcription, its mRNA was gauged at 3 hours post-iron injection (temporally corresponding with the 3-hour plasma MCP-1 increments). As depicted in Figure 3, substantial renal cortical MCP-1 mRNA induction resulted from either intravenous iron sucrose or iron gluconate injection. Thus, these mRNA results (*1*) strengthen the conclusion that parenteral irons can indeed, impact the MCP-1 pathway; and (*2*) imply that the observed plasma and renal cortical MCP-1 elevations likely arise, at least in part, from transcriptional events.

It remained possible that the above described changes reflected only transitory effects, and if so, they could be of limited biologic relevance. Therefore, to gain evidence for a possible more durable response, MCP-1 mRNA levels

were assessed in liver and kidney at both three and seven days post-intravenous iron administration. As shown in Figure 5, by 3 days post-injection, iron sucrose caused three- to fourfold increases in both hepatic and renal cortical MCP-1 mRNA levels. Notably, these elevations were approximately two times greater than those observed at the 3-hour time point, suggesting that increased, not decreased, iron toxicity had occurred. At 1 week post iron gluconate injection, a fourfold increase in renal cortical MCP-1 elevation was still observed. Given that the normal life span of a CD-1 mouse is ~700 days [47], persisting renal MCP-1 mRNA elevations for least 7 days equates with ~1% of the animal's life span (or, equating in human terms, to ~1 year). Clearly, this represents a sufficiently long period to potentially impact the course of most forms of chronic nephropathies. It is notable that iron is sequestered within macromolecular carbohydrate carriers. Thus, as intracellular iron/carbohydrate complex catabolism occurs, progressive iron release may induce increasing and/or persistent oxidative stress, and hence, persistent activation of the MCP-1 axis.

Given that iron injections caused MCP-1 mRNA generation in liver and kidney, the potential for additional organ involvement was sought. This was done to more fully define potential sites which might contribute to the iron-induced plasma MCP-1 elevations. When kidney, liver, lung, spleen, and heart were analyzed simultaneously, quite broad-based induction of MCP-1 message was observed. Thus, despite the fact that the reticuloendothelial system is thought to be dominant site of intravenous iron sequestration, widespread oxidative stress, with resultant MCP-1 induction, appears to be the case. This finding could be particularly relevant for the kidney. Given MCP-1's molecular size of 26.2 kD, ready filtration of circulating MCP-1 could result. Thus, organ "cross talk,' whereby extrarenal MCP-1 generation directly contributes to renal MCP-1 accumulation, might well occur.

In previous studies from this laboratory, we demonstrated that the nature of the carbohydrate polymer used in the different ferric iron preparations can dramatically impact the severity of cell injury sustained. The rank order of that toxicity, iron sucrose > iron gluconate > iron dextran has been consistently observed throughout our past studies, irrespective of the model systems (e.g., in vivo and in vitro) or biologic end points [e.g., cell death and tumor necrosis factor-α (TNF-α) generation] which have been used [36–39]. This rank order of toxicity has directly correlated with, and may be explained by, the degree of cellular iron complex uptake (again, iron sucrose > iron gluconate > iron dextran) [37]. The potential clinical relevance of these experimental observations remains conjectural. However, it seems plausible that the agent with the least experimental toxicity might have a therapeutic advantage, given that all agents appear to be equally effective in supporting erythropoeisis. With these considerations in mind, a further toxicologic comparison of iron sucrose, iron gluconate, and iron dextran was undertaken, using MCP-1 induction at 3 hours post-intravenous injection as the biologic "read-out." Consistent with our previous findings, iron dextran was the least toxic, given that it alone failed to increase either plasma MCP-1, or significantly alter renal/hepatic MCP-1 mRNA. However, in contrast to each of our previous studies [36–39], iron gluconate appeared considerably more toxic than iron sucrose, using MCP-1/mRNA as end points. The reason for this difference between past and present results remains unknown. However, these findings do underscore our previous assertion that not all iron compounds are equivalent in terms of their toxicologic effects. Indeed, a better understanding of the basis for these differential toxicities could potentially help in designing even safer agents for future clinical usage.

The final goal of this study was to assess whether the observed induction of MCP-1 via parenteral iron administration might have potential biologic relevance for the pathogenesis of renal disease. To this end, the impact of iron gluconate on MCP-1 protein generation in an experimental model of progressive nephropathy, urinary tract obstruction, was undertaken. The reason for choosing this model is that complete unilateral ureteral obstruction provides a highly uniform model of injury, thereby allowing accurate assessment of independent iron effects. Furthermore, the presence of a "normal" contralateral kidney (excepting the compensatory changes inherent to a reduction in renal mass) provides each obstructed kidney with its own surgical contralateral control. As shown in Figure 6, by 42 hours post iron gluconate injection, no change in contralateral kidney MCP-1 protein expression was observed (compared to saline-injected surgical controls). Ureteral obstruction, per se, induced modest MCP-1 protein elevations (by ~3 pg/mg). However, this response was drastically increased (by ~12 pg/mg) in obstructed kidneys subjected to iron gluconate pretreatment. Thus, these data support the possibility that iron treatment has a potential to increase inflammatory responses in at least one model of progressive renal disease. Finally, it is notable that intravenous iron increased peritoneal fluid MCP-1 levels by 18 hours after its intravenous injection. This raises a further clinical question. Is it possible that intravenous iron, with resultant peritoneal MCP-1 accumulation, might alter peritoneal mononuclear cell recruitment, and thereby contribute to peritoneal inflammation during maintenance peritoneal dialysis? The above experiments obviously cannot answer these questions. However, they do allow for the generation of some new, and provocative, clinical hypotheses.

It remains unknown as to what cell types within the kidney were responsible for the MCP-1 increases following iron carbohydrate complex exposure. However, it is notable that cultured HK-2 cells responded to iron

gluconate with an increase in MCP-1 expression. These data mirror those obtained by Kanakiriya et al [48] who demonstrated that a chemically dissimilar form of iron (hemin) up-regulated both MCP-1 and its mRNA in cultured rat proximal tubular cells. Thus, these two sets of findings imply that the proximal tubule likely contributes to the in vivo MCP-1 increments noted in the current in vivo experiments. Whether other cell types also participate to the observed cortical increments remains to be resolved.

CONCLUSION

The present experiments provide the following new insights: (*1*) parenteral iron administration has the potential to induce pronounced, and prolonged (≥7 days), stimulation of the MCP-1 axis; (*2*) this process may be particularly pronounced in the presence of renal disease, as evidenced by an approximate fourfold increase in iron-induced MCP-1 accumulation in the setting of at least one model of progressive renal disease (obstructive nephropathy); (*3*) intravenous iron may also induce MCP-1 generation at extrarenal sites (most notably, liver, lung, and spleen) [given its low molecular weight (~26 kD), MCP-1 generated at these extra-renal sites might undergo glomerular filtration, thereby further increasing the intra-renal MCP-1 burden]; and (*4*) not all iron compounds are equivalent in terms of their toxicologic effects. In regards to MCP-1 induction, iron gluconate appears to be the most potent, whereas iron dextran appears devoid of this effect. The reason(s) for these compound-dependent differences remain unknown. However, they underscore the possibility that by altering the nature of the carbohydrate structures, it might be possible to mitigate iron's toxicologic effects. Finally, despite clear differences in toxicologic profiles among iron preparations noted in this and prior studies [36–39], no clinical conclusions vis à vis drug safety/superiority are warranted at this time. Rather, these experimental works should only be used for hypothesis generation, hopefully serving as a stimulus for future clinical investigation into these issues.

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